Standard Operating Procedure:

Lysosomal Acid Lipase (LAL) Enzyme Activity Assay Kit Cat. No. CS2

SOP ID:	SOPPCS2
Version Number:	V3.0
Version Date:	14 April 2026
Product Name:	LAL Enzyme Activity Assay Kit
Matrices:	Dried Blood Spot

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Section 1. Materials and Reagents

Table 1.1: Reagents and Standards

Material	Vendor	Vendor Reference
LAL Enzyme Activity Assay Kit	Enfanos	CS2
MilliQ Purified Water (18.2 MΩ-cm resistivity), or Equivalent	Various	n/a
LC-MS Grade Ethyl Acetate* (CAS: 141-78-6)	JT Baker	9828-03
HPLC Grade Methanol (CAS: 67-56-1)	Fisher	A452
HPLC Grade Acetonitrile (CAS: 75-05-8)	Fisher	A998
ACS Grade Ethanol, 200 Proof (CAS: 64-17-5)	Decon Laboratories, Inc.	2701
Optima Formic Acid (LC-MS) (CAS: 64-18-6)	Fisher	A117
Sodium Chloride (ACS Reagent Grade) (CAS: 7647-14-5)	Sigma Aldrich	S9888

** Note: Trace amounts of oxidizers (e.g., peracetic acid) are present in some grades of Ethyl Acetate due to the manufacturing process. We suggest using the LC-MS grade of Ethyl Acetate available from J.T. Baker, which has been found to be free of this issue. At this point, we cannot guarantee that Ethyl Acetate from this source will always be free of this issue. If the issue presents, you will notice that the MS/MS signal for the product and internal standard reads lower than normal.*

Table 1.2: Equipment and Consumables

Material	Vendor	Vendor Reference
MilliQ Water Purification System	MilliQ	Any System
3.2 mm (1/8") paper punch	Any	Any
Parafilm	Any	Any
Deep-well 96-well plate (polypropylene)	Costar	3959
Silicone mat for 96-well plate	Costar	3080
(optional) 1.5 mL tube with snap cap (polypropylene)	VWR	89000-028
96-well microplate (polypropylene)*	Greiner Bio-One	651201
Zone-Free Sealing Film for 96-well microplate	Sigma	Z721646
Incubator with orbital shaker	Any	Any
Pipette, Single Channel, 2-20 µL (or equivalent)	Any	Any
Pipette, Single Channel, 100-1000 µL (or equivalent)	Any	Any
Pipette, Multichannel, 20-200 µL (or equivalent)	Any	Any
P10, P200, and P1000 Pipette tips	Any	Any
Calibrated Hamilton Syringes for accurate small volume measurement	Hamilton	Any
Centrifuge for 96-well plate (swinging bucket rotor)	Any	Any
96-Well plate nitrogen jet manifold (for use at ambient temperature)	Any	Any
2-8°C Refrigerator (Explosion Proof Recommended)	Any	Any
≤-10°C Freezer	Any	Any
Fume Hood	Any	Any
Needle and Syringe (glass or polypropylene)	Any	Any

** Note: Choose autosampler vials or 96-well autosampler plates that are compatible with the UPLC autosampler that will be used for analysis.*

Section 2. Preparation of the Assay Cocktail

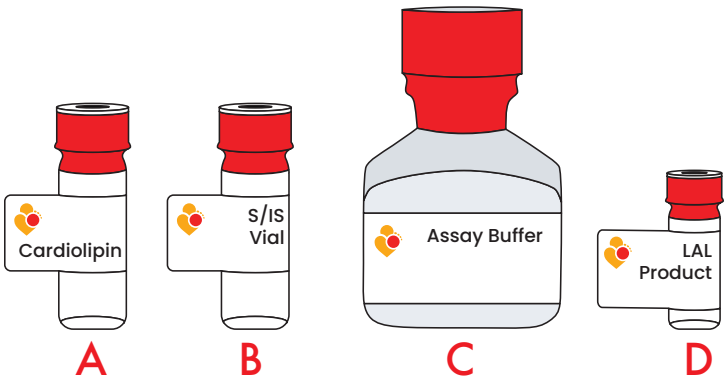


Figure 2.1 Components of the LAL Enzyme Activity Assay Kit

2.1 Instructions for Preparation of the Assay Cocktail

The Assay Buffer (C) is provided by Enfanos as a part of the LAL Enzyme Activity Assay Kit (Cat. No. CS2).

Step 1. Allow the Assay Buffer (C) and S/IS vial (B) to warm to room temperature.

Step 2. The Cardiolipin vial (A) may contain either a solid or a pre-prepared ethanolic solution (5 mg/mL). If the Cardiolipin is received as a solid, prepare the cardiolipin solution by adding 1.0 mL of 200 Proof Ethanol (ACS Reagent Grade) per 5 mg of solid to the vial, then dissolve the solid by vortexing and/or sonication. Ensure the solid is completely dissolved by holding the vial up to a light and inspecting visually for any remaining undissolved material.

Next, add 3 µL of ethanolic cardiolipin solution (A) per assay to the S/IS vial (B) (150 µL for 50 assays) using a Hamilton Syringe or calibrated pipette. Vortex and allow the vial to sit at room temperature for 15-30 minutes, then vortex again until all material is dissolved. The vial should be visually inspected with light behind it to ensure all particulates and/or film on the glass is dissolved. **A crystal clear solution must be obtained before you proceed**.

Step 3. Next, add 27 µL of the Assay Buffer (C) per assay to the S/IS vial (B) (1.35 mL for 50 assays) using a calibrated pipette. Vortex the vial and visually inspect with light behind it to ensure all particulates and/or film on the glass is dissolved and a clear solution is formed.

If you have questions about the composition of the resulting **Assay Cocktail**, please contact us at contact@enfanos.com. Additional components (detergent, inhibitors, activators, buffer salt, etc.) are non-hazardous and are not listed.

Step 4. To prepare a 200 µM methanolic solution of the Enzymatic Product for LC-MS/MS tuning or standard curve preparation, add 5 µL of methanol per 1 nmole of Enzymatic Product in the Enzymatic Product vial (D) using a Hamilton Syringe. Vortex to mix. Prepare additional dilutions as needed in 1:1 (v:v) Water:Methanol. The molecular weight of Enzymatic Product is 218.25 g/mol.

2.2 Storage and Stability of Reagents

Formal stability data for long-term storage of Cardiolipin (A), S/IS Vials (B), Enzymatic Product Vials (D), Assay Buffer (C) are not available.

The Cardiolipin (A) should be stored ≤-10°C. The dry S/IS vials (B) and Enzymatic Product Vials (D) should be stored ≤-10°C. The Assay Buffer (C) should be stored at 2-8°C.

The **Assay Cocktail** (prepared in Section 2.1, Step 3) should be prepared fresh before use. Formal stability data for the **Assay Cocktail** are not available.

Long term storage data on dry reagents (S/IS Vials (B) and Enzymatic Product Vials (D)) are not available. We prefer to refrain from giving artificially short expiration dates, which would lead to wasteful discarding of reagents. However, our knowledge of chemical stability leads us to suggest that the dry reagents are expected to be indefinitely stable when stored at ≤-10°C under dry conditions.

The user is solely responsible for determining suitability of this method for any application.

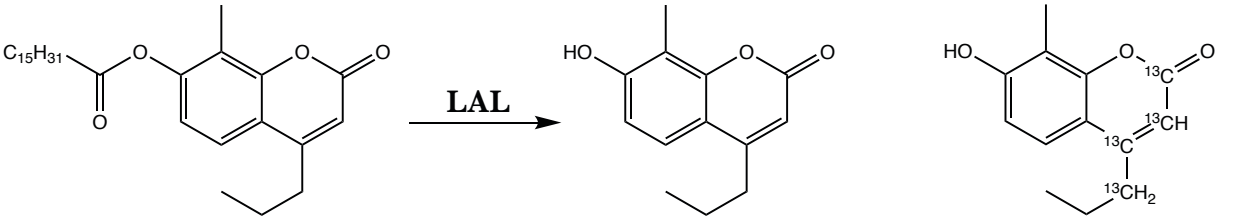
Section 3. Recipes for Other Stocks and Buffers

3.1 Reconstitution Solvent (0.1% Formic Acid in 50:50 (v:v) Water:Acetonitrile)

To prepare 500 mL of Reconstitution Solvent, combine 250 mL of HPLC Grade Acetonitrile and 250 mL of MilliQ Purified Water (or equivalent) in a borosilicate glass or polypropylene solvent bottle. To this, add 0.5 mL of Optima Formic Acid (LC-MS Grade) and mix well. Store at room temperature for up to 6 months.

Section 4. Step-by-Step Method

Figure 4.1 Enzyme Assay Scheme



Exact Mass: 456.32
Molecular Weight: 456.67
LAL Substrate

Exact Mass: 218.09
Molecular Weight: 218.25
LAL Product

Exact Mass: 222.11
Molecular Weight: 222.22
LAL Internal Standard

Step 1. Add one 3.2 mm DBS punch to a well in a 96-well deep-well plate (alternatively, use a 1.5 mL polypropylene microcentrifuge tube) and add 200 µL of MilliQ Water (or equivalent).

Seal the plate with a silicone 96-well plate mat (or cap the tube). Spin the plate (or tube) in a centrifuge at 200g for 10-20 seconds (sec) to bring all the liquid to the bottom, then shake on an orbital shaker set to shake appropriately for 1 hour at room temperature.

** Note: The exact shaking setting will vary depending on the equipment used. The laboratory should determine the optimal shaking speed for their incubator/shaker as part of method implementation.*

Step 2. After DBS extraction, pipet the DBS extracts up and down 5 times, then transfer 10 µL of the liquid to a new deep-well 96-well plate (or new 1.5 mL polypropylene microcentrifuge tube).

Next, add 30 µL of **Assay Cocktail** (prepared in Section 2.1, Step 3) to each well.

Seal the plate with a silicone 96-well plate mat (or cap the tube). Spin the plate (or tube) in a centrifuge at 200*g* for 10-20 seconds (sec) to bring all the liquid to the bottom, then incubate on an orbital shaker set appropriately for 3 hours at 37°C. Record the time when the plate is put on the incubator. This is the start time for the enzyme activity assay incubation.

Step 3. After incubation, spin the plate (or tube) in a centrifuge at 200g for 10-20 seconds to bring all the liquid to the bottom, then quench the reaction by adding 80 µL of MilliQ Water (or equivalent) and 400 µL of LC-MS Grade Ethyl Acetate* (see note in Table 1.1). Pipet the mixture up and down 10 times to mix well.

**Note: only polypropylene or glass materials should be used to dispense Ethyl Acetate, as the solvent dissolves most plastics. Work with Ethyl Acetate should be done in a fume hood.*

Record the time when the MilliQ Water (or equivalent) is added: this is the end time for the enzyme activity assay incubation.

Cover the plate with the silicone 96-well plate mat (or cap the tube) and spin in a centrifuge at 3000g for 5 minutes at ambient temperature to completely separate the aqueous (bottom) and Ethyl Acetate (top) layers.

Step 4. After centrifugation, transfer 120 µL of the top Ethyl Acetate layer to a 96-well polypropylene microplate appropriate for the autosampler you will use.

Evaporate the solvent from each well at room temperature under a nitrogen jet 96-well manifold evaporator.

** Note: If the plate must be stored overnight prior to LC-MS/MS analysis, cover with zone-free sealing film and store at ≤-10°C. On the day of LC-MS/MS analysis, allow the plate to warm to room temperature and proceed to Step 5.*

Step 5. On the same day as LC-MS/MS analysis, add 200 µL of Reconstitution Solvent (see Section 3.1) to each well. Mix with a multichannel Pipette by pipetting up and down 10x.

Seal the plate using zone-free sealing film, or as appropriate for your autosampler, and submit to LC-MS/MS analysis (see Section 5 for recommended method conditions).

Section 5. Recommended Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method

Table 5.1: Materials and Reagents

Material	Vendor	Vendor Reference
Optima Water (LC-MS) (CAS: 7732-18-5)	Fisher	W6500
Optima Acetonitrile (LC-MS) (CAS: 75-05-8)	Fisher	A955
Optima Isopropanol (LC-MS) (CAS: 67-63-0)	Fisher	A461
Optima Formic Acid (LC-MS) (CAS: 64-18-6)	Fisher	A117
CSH C18 Column, 1.7 µm, 2.1 X 50 mm*	Waters	186005296
CSH C18 Pre-column, 1.7 µm, 2.1 X 5 mm*	Waters	186005303
Acquity 2D UPLC System*	Waters	Or Equivalent
Triple Quad Mass Spectrometer	Waters	Or Equivalent

** Note: While Enfanos recommends use of Waters equipment for LC-MS/MS analysis, equivalent instrumentation may be used for analysis of our enzyme activity assay products and internal standards.*

5.1 LC Method

- **Solvent A.** 0.1% Formic Acid in 70:30 (v:v) Water:Acetonitrile
- **Solvent B.** 0.1% Formic Acid in 65:35 (v:v) Isopropanol:Acetonitrile
- **Weak Needle Wash.** 0.1% Formic Acid in 90:10 (v:v) Water: Acetonitrile
- **Strong Needle Wash.** 0.1% Formic Acid in Acetonitrile
- **Column Temperature.** 55°C

Table 5.2: Gradient Program

Time (min)	Flow (mL/min)	Solvent B (%)
initial	0.8	0.5
0.75	0.8	22
1.00	0.8	28
1.50	0.8	40
1.80	0.8	100
2.15	0.8	100
2.20	0.8	0.5

** Note: To minimize contamination of the ESI source, we advise to divert the LC flow to waste except in the retention time region (see Table 5.3) where the product and internal standard elute (~0.8 min).*

5.2 MRM Method

MRM Transitions (see Table 5.3) are measured in ESI positive mode.

** Note: Method developed for analysis on Waters XEVO TQ Instrument (or instrument with equivalent sensitivity). If a higher sensitivity instrument, such as a Waters XEVO TQ-S Micro is being used we recommend adjusting sample preparation so the injected solution contains less moles of analyte or detuning the collision energy of your analytes so the internal standard peak areas are approximately 100,000 AUC. This will ensure linearity throughout the analytical range*

Table 5.3: MRM Method

Analyte	Parent (m/z)	Product (m/z)	Retention Time (min)
LAL-Product	219.1	190.0	0.8
LAL-Internal Standard	223.1	194.0	0.8
LAL-Substrate	458.0	219.0	1.9

** Note: Transitions, cone voltage, collision energy, and retention times need to be confirmed or determined by tuning on your LC-MS/MS instrument in the usual way.*

5.3 Formula for Specific Activity

$$\textbf{\textit{LAL Specific Activity}}\ (\mu\textbf{\textit{mol/h/L}}) = \frac{\boldsymbol{P}}{\boldsymbol{IS}} \times \frac{\mathbf{0.00015}\ \boldsymbol{\mu mole}}{\mathbf{3}\ \boldsymbol{h} \times \frac{\mathbf{1}}{\mathbf{20}} \times \mathbf{3.2} \times \mathbf{10^{-6}}\boldsymbol{L}}$$

To obtain the enzymatic activity, multiply the product-to-internal standard peak area ratio by the micromoles of internal standard in the assay (0.00015 µmol). This number is then divided by the incubation time (3 hours) and 1/20th of the liters of blood in a 3.2 mm DBS punch (3.2 x 10^{-6} L).

** Note: To calculate the incubation time, subtract the enzyme activity assay start time (see Section 4, Step 2) from the enzyme activity assay end time (see Section 4, Step 3).*

*** Note: This procedure describes the preparation and handling of research-use-only materials. It does not establish or support clinical testing, diagnostic use, or patient result interpretation. Any use of this product in laboratory developed tests or clinical applications is the sole responsibility of the user. Enfanos does not validate or support clinical or diagnostic use.*

Section 6. References

References are provided for general scientific background only and do not imply clinical performance, intended use, or validation of this method.

Masi, S., Chennamaneni, N., Turecek, F., Scott, C.R. and Gelb, M.H., 2018. Specific substrate for the assay of lysosomal acid lipase. Clinical chemistry, 64(4), pp.690-696.

Hong, X., Sadilek, M. and Gelb, M.H., 2020. A highly multiplexed biochemical assay for analytes in dried blood spots: application to newborn screening and diagnosis of lysosomal storage disorders and other inborn errors of metabolism. Genetics in Medicine, 22(7), pp.1262-1268.

Section 7. Document Audit Trail

Previous versions of this SOP are stored internally by Enfanos, LLC. If you require a copy of a previous version for comparison to this most recent version, please contact us at contact@enfanos.com